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Title: Analysis of Somatic Hypermutation in the JH4 intron of Germinal Center B cells from Mouse Peyer's Patches

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Yes**

   If **Yes**, can you record movies/images using your own microscope camera? **Yes**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interview Statements are read by JoVE's voiceover talent.

4. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   If **Yes**, how far apart are the locations? **10-minute walk**

Current Protocol Length

Number of Steps: 19
Number of Shots: 47

Introduction

1. Introductory Interview Statements

Videographer: Authors have asked that our VO talent record the introduction and conclusion statements, so you can skip to the protocol.

REQUIRED:

1.1. This protocol allows users to study how the immune system produces antibodies to pathogens or commensal microbes. In addition, molecular pathways that regulate immunoglobulin gene diversification can be studied using genetically engineered mice.

1.1.1. *3.7.2.*

1.2. The protocol uses a combination of common molecular biology techniques, including PCR and Sanger sequencing, and does not require single cell sorting or deep sequencing.

1.2.1. *4.6.1.*

OPTIONAL:

1.3. The PPs can be easily confused with the surrounding fat tissue, so visual demonstration of this method may be helpful for those who are unfamiliar with this organ to identify its location and observe its dissection.

1.3.1. *2.3.2 – 2.4.2.*

Introduction of Demonstrator on Camera

1.4. Demonstrating the procedure will be Emily Sible, a graduate student from my laboratory.

1.4.1. INTERVIEW: Author saying the above.

1.4.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Ethics Title Card

1.5. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at The City College of New York.

Protocol

2. Dissection of Peyer's Patches

2.1. Begin by laying the mouse on the dissection pad with the abdomen exposed **[1]**. Generously spray the body of the mouse with 70% ethanol prior to making any incisions to sterilize the dissection area **[2]**.

2.1.1. WIDE: Establishing shot of talent laying the mouse on the dissection pad.

2.1.2. Talent spraying the mouse with ethanol.

2.2. Make an incision into the skin across the abdomen **[1]** and pull simultaneously on both sides of the incision with forceps **[2]**. Pin down the fore and hind limbs of the mouse **[3]**, then cut the peritoneal cavity with scissors to expose the internal organs **[4]**.

2.2.1. Talent making the incision in the abdomen.

2.2.2. Talent pulling the skin apart.

2.2.3. Talent pinning down the limbs of the mouse.

2.2.4. Talent cutting the peritoneal cavity.

2.3. Locate the small intestine and remove it by cutting below the stomach and above the caecum **[1]**. Remove any connective tissue and fat linking the folds of the small intestine together **[2]**. *Videographer: This step is important!*

2.3.1. Talent cutting out the small intestine.

2.3.2. Talent removing the connective tissue and fats.

2.4. Examine the external surface of the small intestine for the Peyer's patches **[1]**, which are small, oval-shaped structures that appear white below a thin layer of translucent epithelial cells **[2]**. *Videographer: This step is important!*

2.4.1. Talent looking for Peyer's patches.

2.4.2. SCOPE: A Peyer's patch. NOTE: Author thinks that videographer submitted "footage from his camera" for this one.

2.5. Carefully excise all visible Peyer's patches with scissors **[1]** and collect them into a 1.5-milliliter microcentrifuge tube containing 1 milliliter of FACS buffer on ice **[2]**. If the collected tissue sinks to the bottom of the tube, it is in fact a Peyer's patch and not fat tissue **[3]**. *Videographer: This step is difficult and important!*

2.5.1. Talent excising PPs.

2.5.2. Talent putting a PP into the collection tube on ice.

2.5.3. ECU: Peyer's patch sinking at the bottom of the tube.

3. Cell Isolation and Staining for FACS

3.1. Place a 40-micrometer filter in a 6-well dish with 1 milliliter of cold FACS buffer **[1]**, then pour the Peyer's Patches from the 1.5-milliter tube onto the filter **[2]**. Use the flat end of the plunger from a 1-milliliter syringe as a pestle to crush the Peyer's Patches on the filter until only the connective tissue remains **[3]**. *Videographer: This step is important!*

3.1.1. Talent putting a filter in the 6-well dish.

3.1.2. Talent pouring the PPs on the filter.

3.1.3. Talent pushing the PPs through the filter.

3.2. Wash the filter and plunger with 1 milliliter of cold FACS buffer to release the cells into the 6-well dish **[1]**. Collect the cells and filter them through a 40-micrometer strainer cap FACS tube **[2]**, then wash the strainer cap with 1 milliliter of cold FACS buffer **[3]**. *Videographer: This step is difficult and important!*

3.2.1. Talent washing the filter and plunger.

3.2.2. Talent filtering the cells through the strainer cap.

3.2.3. Talent washing the strainer cap with FACS buffer.

3.3. Pellet the cells by centrifuging them at 600 *x g* at 4 degrees Celsius for 5 minutes **[1]**, then decant the supernatant **[2]** and resuspend the cells in 0.4 milliliters of cold FACS buffer with Fc block **[3]**. Incubate samples on ice for 15 minutes, then wash and pellet the cells **[4]**.

3.3.1. Talent putting the cells in the centrifuge and closing the lid.

3.3.2. Talent decanting the supernatant.

3.3.3. Talent resuspending the cells.

3.3.4. Talent putting the cells on ice.

3.4. To stain the germinal center B cells, or GCBCs, resuspend the wildtype cells in 80 microliters of FACS buffer. Remove 10 microliters of cells from the wild type Peyer's Patches for each staining control, leaving 40 microliters of the cell suspension for the experimental samples. Alternatively, use compensation beads for the staining controls **[1-TXT]**. *Videographer: This step is important!*

3.4.1. Talent aliquoting the cell suspension for staining controls. **TEXT: 4 total, 3 single stain and 1 unstained**

3.5. Stain each of the experimental samples in 500 microliters of cold FACS buffer with 2.5 microliters of PNA-biotin for 15 minutes on ice **[1-TXT]**, then wash the PNA-stained cells **[2]**. Stain each sample with 500 microliters of GCBC cocktail, in the dark and on ice, for 15 minutes, making sure that the cells are fully resuspended in the cocktail **[3]**.

3.5.1. Talent adding PNA-biotin to a sample on ice. **TEXT: PNA: peanut agglutinin**

3.5.2. Talent washing the cells.

3.5.3. Talent adding the staining cocktail to a sample on ice. Video Editor: Show Table 1 as an inset here.

3.6. To prepare the single stain controls for the compensation matrix, stain the cells in 500 microliters of cold FACS buffer using the dilutions specified in the text **[1]**. Incubate the staining controls in the dark, on ice, for 15 minutes **[2]**.

3.6.1. Talent adding staining solutions to the control cells.

3.6.2. Talent putting the cells in a dark place to incubate.

3.7. Add 2 milliliters of cold FACS buffer to wash all the stained samples and controls. Pellet the cells and discard the supernatant before resuspending the cells in a final volume of 500 microliters of cold FACS buffer to run on the cytometer **[1]**. Then, use a cell sorter to collect the $B220^{+}PNA^{HI}$ *(pronounce 'B-two-twenty-positive-PNA-high)* cells from each stained experimental sample **[2]**.

3.7.1. Talent taking a sample with pelleted cells out of the centrifuge.

3.7.2. Talent using the cell sorter.

4. JH4 Intron Sequence Amplification and Analysis

4.1. To extract the DNA from the GCBCs, resuspend the cells in 500 microliters of DNA extraction buffer and 5 microliters of Proteinase K **[1-TXT]**. Then, precipitate the DNA with 500 microliters of isopropanol and 1 microliter of glycogen **[2-TXT]** and mix the tube thoroughly by inverting it 5 or 6 times **[3]**.

4.1.1. Talent adding extraction buffer and proteinase K to the cells and resuspending them. **TEXT: 20 mg/mL Proteinase K**

4.1.2. Talent adding isopropanol and glycogen to the sample. **TEXT: 20 mg/mL glycogen**

4.1.3. Talent inverting the tube a few times.

4.2. After incubating the sample for 10 minutes at room temperature, centrifuge it for 15 minutes at 21,000 *x g* and 25 degrees Celsius **[1]**. Discard the supernatant **[2]** and wash the DNA pellet with 1 milliliter of 70% ethanol **[3]**. Centrifuge the DNA at 21,000 *x g* for 10 minutes, then remove the ethanol and air-dry the pellet for 5 to 10 minutes **[4]**.

4.2.1. Talent putting the samples in the centrifuge and closing the lid.

4.2.2. Talent discarding the supernatant.

4.2.3. Talent adding ethanol to the DNA.

4.2.4. Talent removing ethanol from the centrifuged sample.

4.3. Resuspend the DNA in 30 microliters of TE buffer **[1]** and incubate it overnight at 56 degrees Celsius **[2]**.

4.3.1. Talent resuspending the DNA in TE buffer, with the buffer container in the shot.

4.3.2. Talent putting the sample in the water bath and closing the lid.

4.4. Perform nested PCR for the JH4 intron, normalizing the total amount of genomic DNA used in the first PCR to the least concentrated sample **[1]**. After running the PCR products on a gel, excise the amplicon and purify it with a gel extraction kit **[2]**.

4.4.1. Talent putting PCR tubes in the thermocycler.

4.4.2. Talent excising amplicon from a gel.

4.5. Then, ligate the amplicon into a plasmid and transform electrocompetent bacterial cells with 2 microliters of the ligation reaction **[1]**. Electroporate the cells at 1.65 kilovolts **[2]** and rescue them in 600 microliters of SOC media for 1 hour at 37 degrees Celsius in a shaking incubator at 225 rpm **[3]**.

4.5.1. Talent adding plasmid to bacteria.

4.5.2. Talent electroporating the bacteria.

4.5.3. Transformation tubes with cells and media shaking in the incubator.

4.6. Plate 100 microliters of the transformed bacteria onto LB agar plates supplemented with ampicillin **[1]** and incubate the plates overnight at 37 degrees Celsius **[2]**.

4.6.1. Talent spreading the bacteria on a plate.

4.6.2. Talent putting the plates in the incubator and closing the door.

4.7. Sequence the plasmid DNA from the bacterial colonies and align the sequences obtained for each PCR against the JH4 intron reference sequence using a Clustal Omega software. Identify differences from the reference sequence as mutations **[1]**.

4.7.1. Talent at the computer comparing sequences.

Results

5. Results: Flow Cytometry and JH4 Sequence Analysis

5.1. The GCBCs were identified by measuring expression of the CD45R-B220 receptor and binding of peanut agglutinin **[1]**. Wild type Peyer's patches contained an average of 4 million cells per mouse **[2]**, and approximately 8% of the cells were $B220^{+}PNA^{HI}$ *(pronounce 'B-two-twenty-positive-PNA-high)* **[3]**, which is half of that observed in $AID^{-/-}$ *(spell out 'A-I-D' knockout)* mice **[4]**.

5.1.1. LAB MEDIA: Figure 3 A and B.

5.1.2. LAB MEDIA: Figure 3 A and B. *Video Editor: Emphasize A.*

5.1.3. LAB MEDIA: Figure 3 B. *Video Editor: Emphasize the WT data.*

5.1.4. LAB MEDIA: Figure 3 B. *Video Editor: Emphasize the $AID^{-/-}$ data.*

5.2. Of the 105 unique sequences obtained from wild type GCBCs, a total of 226 mutations were found. Analysis of the mutation spectrum showed a range of transitions and transversions at a rate of 4×10^{-3} mutations per base pair **[1]**. Only two mutations were identified in 113 $AID^{-/-}$ sequences **[2]**.

5.2.1. LAB MEDIA: Figure 5 A. *Video Editor: Emphasize the WT plot.*

5.2.2. LAB MEDIA: Figure 5 A. *Video Editor: Emphasize the $AID^{-/-}$ plot.*

5.3. Additionally, each JH4 PCR product from wild type GCBCs contained 1 to 25 mutations, with multiple mutations frequently found on one sequence **[1]**.

5.3.1. LAB MEDIA: Figure 5 B.

Conclusion

6. Conclusion Interview Statements

6.1. When attempting this protocol, remember to keep the cells on ice to preserve cell viability. After staining the cells, keep the cells in the dark to prevent photobleaching the fluorophores.

6.1.1. *2.5.2, 3.6.2.*

6.2. To correlate SHM with antibody affinity maturation, we recommend using an immunization protocol and assaying for antigen-specific antibody titers and V region mutations.

6.2.1. *4.7.1.*